TREATMENT OF PANCREATIC DISEASE

This is a 371 of PCT/AU00/01026 filed 6/30/00

Field of Invention

The present invention relates to treatment of pancreatic diseases of humans

5   and animals. This invention has particular but not exclusive application for treatment

of acinar cell carcinoma, mixed cell (including acinar cell) pancreatic carcinoma,

acute and chronic pancreatitis.

Prior Art

10   The pancreas is a secretory gland comprising approximately 80% of acinar

cells, 1% to 2% of islet cells in clusters, and 10% to 15% of single layered cuboidal

ductal cells interlaced with blood vessels, lymphatics, nerves, and collagenous

stroma (Evans et al. Cancer of the Pancreas. In Cancer: Principles and Practice of

Oncology, 5th edition, DeVita et al. (Eds), Lippincott-Raven, New York).

15   Despite the large population of acinar cells, acinar cell carcinoma only

accounts for 1% - 3% of pancreatic carcinomas. In addition only 5% - 10% of

pancreatic carcinomas comprise mixed cell populations including acinar cells

(Nonomura et al., 1992 Ultrastructural Pathology 16:317-329; Cubilla and Fitzgerald

1975 Cancer Research 35:2234-2248). Pancreatic mixed cell carcinomas and acinar

20   cell carcinomas have been reported to be aggressive diseases with a high fatality

rate (Klimstra et al., 1992, Am. J. Surg. Pathol. 16(9):815-837; Adis Editors. 1997,

The Oncology Review 2-4). Surgical resection is the recommended treatment for

pancreatic carcinomas. However even with reductions in operative mortality

little from its initial description in 1935 with the current 5 year survival rate being

between 2% and 5% (Adis Editors. 1997, The Oncology Review 2-4).

Another disease of the pancreas is acute pancreatitis which appears to have

variable severity. Acute pancreatitis appears to arise when the pancreatic duct is

5   obstructed by a gallstone or tumour, or when toxins to the pancreas such as ethanol

are ingested. Enzyme production continues causing digestion of the pancreas with

often fatal results. Recurrent bouts of acute pancreatitis (or chronic pancreatitis)

often result in scarring and deformation of the ductal system thereby causing

localised obstruction and thus perpetuating pain, disability and digestive deficiency.

10   The events which regulate the severity of acute pancreatitis are unknown.

Several studies, however, have shown that mild pancreatitis was found to be

associated with extensive apoptotic acinar cell death while severe pancreatitis was

noted to involve extensive acinar cell necrosis but very little acinar cell apoptosis

(Kaiser et al. 1995, Am. J. Physiol. 269:C1295-C1304: Gukovskaya et al. 1996

15   Gastroenterology 110:875-884).

Administration of cyanohydroxybutene (CHB) to a subject appears to affect

inter alia the pancreas. CHB is a glycosinolate breakdown product found in

cruciferous vegetables, raw canola, and many stock feeds. It was observed that

CHB administered at a daily dose of 200 mg/kg to rats by gavage for four days

20   caused acinar cell apoptosis, inflammation, and exocrine pancreatic atrophy (Wallig

et al. 1988, Fd Chem Toxic 26:137-147).

Histological and ultrastructural evaluations have been conducted on rats at

different time periods after administration by gavage of 200 mg CHB/kg body weight

in corn oil. These investigations revealed that as early as 4 hours after CHB

25   administration, the pancreas exhibited abnormal pathology including mild to

moderate supranuclear vacuolation of acinar cells. After 24 hours of CHB

administration, the rats exhibited acinar cell apoptosis with cytoplasmic basophilia,

lack of zymogen, diffuse vacuolation, clumping of chromatin, and nuclear pyknosis or

karyorrhexis (Wallig and Jeffery 1990, Fund. Appl. Toxicol. 14:144-159).

5   Synthetic CHB being racemic mixture of the R- and S- enantiomers

administered by gavage in olive oil at doses of 25-200 mg/kg body weight causes

similar effects in the pancreas of rats as naturally occurring CHB. It was observed

that pancreatic edema and acinar cell vacuolation and depletion of zymogen granules

occurred within hours of administration (Maher et al. 1991, Pancreas 6:168-175).

10   A single dose of 50 mg CHB/kg was administered intravenously to rats and

found to form apoptotic bodies in the pancreas whereas a single dose of 100mg

CHB/kg was found to cause severe pancretotoxicity with necrosis (Wallig et al. 1992

Fundamental Applied Toxicology 19:598-606).

In a further study the relationship between acinar cell apoptosis and the

15   severity of pancreatitis was investigated by administering a single intravenous dose

of CHB (70mg/kg) in corn oil to mice and inducing pancreatitis at varying times after

CHB administration. They found that the severity of pancreatitis is reduced when the

disease is induced during the period in which apoptosis is most extensive. However

induction of pancreatitis either before or after the peak period of apoptosis results in

20   pancreatic injury which is similar to that noted in animals not exposed to CHB (Bhatia

et al.1998, Biochem. Biophys. Res. Commun. 246:476-483). It appears that the

acinar cells may regenerate after treatment with CHB.

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Summary of the Invention

The present invention arises from the surprising discovery that subcutaneous injection of CHB produces an unusual and unsuspected result of acinar cell apoptosis of normal acinar cells and acinar cell carcinoma. It was found that

5   subcutaneous injection of CHB at an appropriate sub-lethal dosage caused apoptosis of the substantially entire population of acinar cells. The pancreatic lesion was unusual in that there was observed a marked early edema with limited inflammatory infiltration, rapid synchronous onset of acinar cell apoptosis and advanced atrophy with only a severely limited regenerative response. The application of this discovery

10   to treat acinar cell carcinoma has led to the development of the current invention.

The present invention in one aspect broadly resides in a method of providing selective, substantially total, non-regenerative apoptosis of pancreatic acinar cells comprising a single-dose, subcutaneous or intra-arterial administration of a composition of cyanohydroxybutene and a pharmacologically acceptable aqueous

15   carrier

An amount within the therapeutic window is the amount below a lethal dose that has the desired therapeutic effect. Preferably, the therapeutic window is selected whereby CHB serum levels are maintained at a level and for a period sufficient to cause apoptosis of substantially the whole acinar cell population, whilst

20   remaining below the threshold serum levels that cause undesirable levels of liver damage.

The patient may be selected whereby the acinar cells being treated include acinar carcinoma cells. The acinar carcinoma cells may be presenting as localized to the pancreas or as metastases.

25   The administration is preferably subcutaneously, intramuscularly or intra-arterial catheter direct to the pancreas. These modes of administration appear to

provide CHB in the blood at a more even rate to form a concentration plateau and

substantially reduce the height of the concentration peak of CHB. Oral and

intravenous administration appears to produce concentration CHB peaks. Oral and

intravenous administration of high doses of CHB causes substantial damage to the

5   liver.

In a further aspect, this invention resides broadly in a method for treating

pancreatic disease including administering to a patient a therapeutically effective

amount of cyanohydroxybutene to cause substantially non-regenerative apoptosis of

acinar cells in the patient.

10   The invention in another aspect resides broadly in a method of treating

pancreatic carcinoma having acinar cells including

preparing a cyanohydroxybutene (CHB) formulation; and

administering one or more sub-lethal doses of the CHB formulation to a

subject with acinar cell carcinoma, wherein the treatment causes substantially non-

15.   regenerative apoptosis of malignant acinar cells in a patient.

The CHB formulation is preferably a CHB solution wherein CHB is

substantially dissolved in water. The CHB may be a natural or synthetically derived

CHB.

The CHB dose is preferably calculated within a range of 5-300mg CHB/kg of

20   body weight. Preferably the CHB dose is within the range of 125-160 mg CHB/kg of

body weight. More preferably the CHB dose is approximately 150 mg CHB/ kg of

body weight. The dosage may vary between subjects. Subjects include animals and

people of different sizes and weight.

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Preferably only one dose is administered. However a second or subsequent dose may be administered but preferably after a period of time such as a week or when the glutathione levels have returned to approximately normal levels.

Administration is preferably by means where the CHB is absorbed

5   comparatively slowly and thus substantially avoids the development of CHB serum concentration peaks that cause undesirable levels of damage to the liver. Preferably the CHB dose is administered by subcutaneous injection, intramuscularly or intra-arterial catheter direct to the pancreas. In an alternative form the CHB dose is administered so that it is delivered directly to the acinar cells. In this form the CHB

10   molecule may be conjugated to a ligand molecule which is able to bind to an acinar cell surface receptor thereby delivering CHB to the acinar cell.

Acinar cell carcinoma includes carcinomas of only acinar cells or of mixed cell populations with a portion being acinar cells.

The above method of treating acinar cells may be applied to the treatment of

15   acute and chronic pancreatitis. Thus in another aspect the present invention broadly resides in a method for treating acute or chronic pancreatitis including

preparing a cyanohydroxybutene (CHB) formulation; and

administering one or more sub-lethal doses of the CHB formulation to a subject with acute or chronic pancreatitis wherein the treatment includes an amount

20   of CHB that causes apoptosis of substantially all acinar cells and substantially no regeneration of acinar cells.

The description of the features of the method for treating acinar cell carcinoma apply also to the above method where applicable.

Brief description of the Drawings

In order that the invention may be more readily understood reference will now

be made to the accompanying drawings which illustrate the experimental results and

a preferred embodiment of the invention and wherein:

5   Figure 1 shows body weights of animals as a percentage of starting weight

after a single subcutaneous injection of saline or CHB (n=4, results expressed as

means ± SEM.) over time after treatment;

Figure 2 shows pancreatic weight as a percentage of body weight in animals

after a single subcutaneous injection of saline or CHB (n=4, results expressed as

10   means ± SEM.) over time after treatment;

Figure 3 shows pancreatic morphology after a single subcutaneous injection of

saline of CHB. All H and E stained. **(A)** 48 hours after saline. There is wide

separation of ducts (arrows) and islets (I) by closely-packed acinar cells (x500). **(B)**

12 hours after CHB. Note numerous apoptotic acinar cells with characteristic nuclear

15   morphology (arrow) (x1200). **(C)** 18 hours after CHB. Most acinar cells contain

pyknotic nuclear remnants (arrowheads) and show cytoplasmic swelling and

vacuolation; a few appear normal (arrows) (x900). **(D)** 48 hours after CHB.

Advanced secondary necrosis affecting all acinar cells in field. Intact duct is

indicated by arrow (x360). **(E)** 96 hours after CHB. No acinar cells remain. Atrophic

20   lobules comprise crowded ducts in a connective tissue stroma. (x200). **(F)** 28 days

after CHB. Sparse regenerative acini are seen adjacent to islets (arrows). Note few

ducts in a collagenous stroma and prominent fatty infiltration, (x200);

Figure 4 shows pancreatic immunohistochemistry after a single subcutaneous

injection of saline or CHB. All with haematoxylin counterstain. **(A)** 24 hours after

25   saline. Widely-spaced keratin-positive ducts (arrows) are separated by closely-

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packed keratin negative acinar cells (x160) **(B)** 48 hours after CHB. Widely-spaced

keratin-positive ducts (arrows) separated by keratin-negative nonviable acinar cells

(x180). **(C)** 96 hours after CHB. Lobules comprise crowded keratin-positive ducts

separated by loose connective tissue. No acinar cells are seen, (x180). **(D)** 96 hours

5   after CHB. Ducts are negative for amylase. Note isolated amylase positive epithelial

cell (arrow) and perinsular amylase positivity (x160);

Figure 5 shows pancreatic ultrastructure after a single subcutaneous injection

of CHB. **(A)** 12 hours after CHB. Adjacent apoptotic acinar cells show well-

demarcated crescentic clumped chromatin, large nucleolar remnants (Arrowhead)

10   and whorling of endoplasmic reticulin (arrows) (x3000). **(B)** 18 hours after CHB.

Apoptotic acinar cells show nuclear fragments with crescentic clumped chromatin but

dilation of endoplasmic reticulin, swollen mitochondria (arrowheads) and plasma

membrane rupture (arrow). Contrast with adjacent viable acinar cells (x2800). **(C)**

48 hours after CHB. Note viable duct epithelial cells (D), residual acinar cell

15   cytoplasmic debris (A) and intra-acinar macrophage (M) laden with residual bodies.

The pale cell in the duct epithelium (arrow) is also likely to represent an intraepithelial

macrophage (x350). **(D)** 96 hours after CHB. Duct with typical indented nuclei and

sparse organelles of lining cells. Note mitotic lining cell (M), intraepithelial apoptotic

body (arrow), and adjacent collapsed redundant basement membrane (arrowhead)

20   (x2500). **(E)** 96 hours after CHB. Activated and mitotic interstitial fibroblasts.

(x.3300). **(F)** 48 hours after CHB. Capillary with intraluminal apoptotic bodies of

presumed endothelial cell origin (arrow). Note adjacent intraacinar macrophage (M)

with residual body-laden cytoplasm (x.7000);

Figure 6 shows **(A).** Untreated AR42J acinar cell carcinoma in an athymic rat,

25   showing broad sheets of cells with an area of haemorrhagic necrosis (arrow) (x100).

(B) Untreated AR42J acinar cell carcinoma, (x400), showing multiple mitoses. (C) AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing widespread apoptosis and an area of surviving cells (arrow). (x100).

(D) AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of

5   140mg/kg CHB, showing widespread apoptotic change (x400).

Figure 7 shows (A) Untreated AR42J acinar cell carcinoma, showing primitive acini formation (x1000). (B) AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing pyknotic and fragmented nuclei and secondary necrosis of cytoplasm (x1000). (C) Pancreas of a normal Wistar rat 24

10   hours after 140mg/kg subcutaneous CHB showing almost total acinar cell death via apoptosis with secondary necrosis (x400). (D) Pancreas of athymic rat 24 hours after 140mg/kg subcutaneous CHB showing regional effect with necrotic areas adjacent to well-preserved areas with pyknotic nuclei (x400).

Fig.8 shows an electronmicrograph of apoptotic cells showing condensed

15   marginated chromatin.

Experimental

1.   Determination of Acinar Cell Apoptosis and Regeneration of Acinar Cells with Subcutaneous Administration of CHB.

20   1.1   Material and Methods

Synthetic CHB made according to the method of Das and Torssell (Das and Torssell 1983 Tetrahedron 39:2243-2247).

Male Wistar rats weighing 200 – 250 g were caged in pairs and given food and water ad libitum with a 12 hours light-dark cycle. Twelve groups of 10 rats were

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divided randomly into 6 test animals and 4 control animals. At time 0, test animals

were given 150mg/kg of CHB mixed in 0.5ml sterile normal saline and controls were

given 0.5ml sterile normal saline subcutaneously.

For light microscopy, 4 experimental and 4 control animals were killed at 2, 4,

5   6, 12, 24, 48, 72 and 96 hours and 7, 10, 18 and 28 days using 60 mg intraperitoneal

pentobarbitone. Animals were weighed and the pancreas removed, weighed and

processed using routine methods. Additional pairs of experimental animals were

killed at 18 and 60 hours for electron microscopy and morphological study. Weights

were recorded as means ± standard error of the mean (SEM). Differences between

10   means were analysed using Student's t-test.

For quantification of apoptosis, apoptotic cells and bodies, identified using the

morphological criteria (Kerr et al. 1995 Method Cell Biol. 46:1-27) and were counted

in ten high-power graticule fields (HPF), selected at random, in a histological slide

from each animal at 2, 4, 6 and 12 hours with the proviso that mostly acinar tissue

15   filled the field. A group of tightly clustered apoptotic bodies, presumably derived from

a single cell, was recorded as a single count. An estimate of the total number of

acinar cells per HPF in each slide was made for calculation of an apoptotic index

(apoptotic count as a percentage of total acinar cells present). Counts/HPF and

apoptotic indices were recorded as means ± SEM for each group. Differences

20   between means were analysed using Student's t-test. Terminal d-UTP nick-end

labelling (TUNEL) was not used because it is our experience and the experience of

others that it is not always specified for apoptosis, and ultimately, apoptosis must be

confirmed morphologically (Ansari et al. 1993 J. Pathol. 170:1-8; Grasl-Kraupp et al.

1995 Hepatology 21:1465-1468).

Immunohistochemistry for cytokeratin an amylase was performed to identify

cells in sections as duct (Schussler et al. 1992 Am. J. Pathol 140:559-568; Bouwens

et al. 1995 J Histochem Cytochem. 43:245-253) or acinar (Bendayan 1984

Histochem J. 16:85-108) respectively. For cytokeratin, deparaffinized sections were

5   pretreated with 0.1% trypsin, then 0.3% hydrogen peroxide in methanol followed by

mouse monoclonal AE1/AE3 anti-cytokeratin at a dilution of 1/40. Secondary

antibody was rat anti-mouse biotinylated IgG used at a dilution of 1/400. Antibody-

binding was demonstrated using the peroxidase-labelled streptavidin biotin complex

method and reactions were developed with 3,3'-diaminobenzidine tetrahydrochloride

10   solution. For amylase, deparaffinized sections were boiled in Target Retrieval

Solution then placed in 0.3% hydrogen peroxide in methanol. Primary antibody was

anti-rabbit immunoglobulin used at a dilution of 1/500 and secondary antibody was

anti-rabbit goat biotinylated IgG used at a dilution of 1/400. Antigen-binding was

demonstrated using the peroxidase-streptavidin method developed with Vector VIP

15   peroxidase substrate. All sections were lightly counterstained with hematoxylin.

For electron microscopy, two rats from each test group were deeply

anaesthetised with intraperitoneal sodium pentobarbitone. A catheter was inserted

into the abdominal aorta and the vasculature flushed in sequence with 1) heparinized

normal saline, 2) 1% paraformaldehyde and 1.2% glutaraldehyde in cacodylate buffer

20   and 3) 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (Karnovsky

1965 J, Cell Biol.27:137A-138A). Pancreas was removed immediately, diced and

immersed in perfusate no. 3 for two hours, then stored in cacodylate buffer. The

tissue was postfixed in 1% osmium tetroxide, stained en bloc in 5% aqueous uranyl

acetate, dehydrated through a series of graded alcohols, cleared in propylene oxide,

25   and embedded in an epon-araldite mixture. Semithin sections (1μm) were cut on an

LKB Ultratome V and stained with toluidine blue for viewing. Ultrathin sections from selected areas were picked up on uncoated copper grids, stained with lead citrate and examined with a JEOL-1200 EX11 electron microscope.

5   1.2   Results

1.2.1 General Observations

Control rats showed no behavioural change and normal weight gain reaching 180% at 28-days (Figure 1). Experimental rats showed discomfort 30 minutes after injection, lost curiosity and became reluctant to move. Body weight fell over the first

10   week and thereafter remained unchanged (Figure 1).

At autopsy, control rats had normal viscera and a pancreatic weight which was constant as a proportion of body weight (Figure 2). Experimental animals showed pancreatic edema from 2 hours, actual pancreatic weight reaching 4.41 ± 0.71 g at 6 hours (compared to 0.78 ± 0.14g in controls, $P<0.001$), then falling. Atrophy was

15   apparent at 7 days and persisted, actual pancreatic weight falling to 0.44 ± 0.04g at this time compared to 1.80 ± 0.08g in controls, $P<0.001$.

Changes in pancreatic weight as a percentage of body weight are shown in Figure 2. At 18 and 28 days CHB-treated rats had muscle wasting, abdominal distension and dilated bowel containing undigested food.

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1.2.2 Light Microscopy

Control animals showed histologically normal pancreas (Figure 3A). From 2 hours test animals showed mild dilation of acinar lumens and acinar cell vacuolation and depletion of zymogen granules.

Apoptotic acinar cells, evident at 6 hours, showed sharply-defined crescents of

clumped chromatin against the nuclear envelope but infrequent fragmentation. Their

number reached 178 ±10/HPF at 12 hours (compared to 0.85 ±0.13/HPF in controls,

$P<0.001$) or 23.6±7.43% of acinar cells (compared to 0.001% in controls) (Figure 3

5 B). By 18 hours most acinar cells had chromatin changes of apoptosis but swollen

vacuolated cytoplasm indicative of "secondary necrosis" (Figure 3C) which

subsequently progressed (Figure 3D). By 96 hours no acinar cells remained (Figure

3E). A few regenerative acini appeared by 18 days, particularly adjacent to islets of

Langerhans, but thereafter they did not increase appreciably in number (Figure 3F).

10 Intercalated ducts were mildly dilated at 4 hours, duct cell mitoses were

prominent at 48 hours, and at 96 hours, lobules comprised groups of ducts within a

connective tissue stroma (Figure 3E). Small numbers of apoptotic bodies continued

to be seen within duct lumens and epithelium. By 7 days ducts had larger lumens

and flattened lining epithelial cells. Thereafter the number of ducts decreased with

15 few remaining at 18 and 28 days (Figure 3F).

Interlobular edema was present from 2 hours and interlobular edema from 4

hours; both persisted for 72 hours. The interstitial spaces were acellular before small

numbers of mononuclear phagocytes appeared about vessels at 4 hours and within

acini at 24 hours. They reached moderate numbers at 48 hours, peaked at 72 hours,

20 then declined markedly by 7 days. Sparse neutrophils were present from 12 hours

and mitotic mononuclear phagocytes at 48 hours.

Enlarged mitotically active fibroblasts were seen 48 hours, by 96 hours

fibroblasts and collagen enveloped lobules and at 7 days fibroblasts were less

prominent and collagen was found both in and around lobules. At 28 days the

25 pancreas comprised largely fat, collagen and islets (Figure 3F).

Islets were not studied in detail. Given the degree of atrophy, however, less islet tissue was apparent than might be expected from simple condensation.

1.2.3 Immunohistochemistry

5   In controls ducts were positive for cytokeratin and acinar cells negative (figure 4A). At 48 hours in test animals, when few viable acinar cells remained, cytokeratin marked dispersed intact ducts and duct cells (Figure 4B). At 96 hours ducts of atrophic lobules, the only remaining epithelium, were positive for cytokeratin (Figure 4C). Amylase was demonstrated in apoptotic cells at 18 hours, confirming their

10   acinar cell origin. There were no or rare amylase-containing cells at 72 and 96 hours (Figure 4D) with occasional apoptotic bodies staining for amylase. The periphery of islets also showed amylase staining at 96 hours (Figure 4D).

1.2.4 Electron Microscopy

15   Controls showed normal pancreatic ultrastructure (Ekholm et al. 1962 J. Ultrastruct. Res. 7:61-72; Ekholm et al. 1962 7:73-83). In test animals acinar cell apoptosis was slightly increased at 6 hours and markedly increased at 12 hours, when large numbers of adjacent cells were often affected (Figure 5A). Apoptotic cells showed sharply-defined crescents of chromatin abutting the nuclear envelope,

20   prominent nuclear remnants, whorling of endoplasmic reticulum and structural preservation of organelles (figure 5A) but cellular fragmentation to form apoptotic bodies was uncommon. At 18 hours, apoptotic cells, identified by their nuclear characteristics, remained in situ, but showed dilation of endoplasmic reticulum and nuclear envelopes, swelling and rupture of mitochondria and rupture of plasma

25   membranes (Figure 5 B), so-called "secondary necrosis". This process progressed

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such that, by 48 hours, acinar cells were reduced to degraded cellular material associated with small number of intraepithelial macrophages containing ingested apoptotic bodies, degraded cellular material in phagosomes of residual bodies (Figure 5C). By 96 hours, acinar cell debris had been removed (Figure 5D). Ducts

5   and duct cells survived, showing increased mitotic activity, particularly at 60 and 72 hours (Figures 5C and D). Small numbers of ductal intraepithelial apoptotic bodies and surrounding collapsed basement membrane were identified (Figure 5 D).

From 48 to 96 hours, prominent activated and mitotic fibroblasts were seen (Figure 5E). At first collagen was sparse but increased in amount towards 7 days. At

10   48 hours, mitoses in interstitial macrophages were confirmed and endothelial cell apoptosis was present in interstitial capillaries (Figure 5F); this continued over succeeding days. By 18 days, isolated regenerative acini comprised acinar cells closely resembling acinar cells in control glands.

15   1.3   Discussion

Within 12 hours of administration of CHB, there is relatively synchronous onset of apoptosis in the majority of acinar cells. This contrasts with the slow onset of apoptosis and gradual increase peaking about the third day that occurs after duct ligation (Walker 1987 Am. J. Pathol. 126:439-451) or the administration of cerulein

20   (Fujimoto et al. 1997 Digestion 58:421-430) or ethionine and a protein-depleted diet (Walker et al. 1993 Pancreas 8:443-449). The sequence is similar but delayed after administration of a copper-depleted diet (Rao et al. 1993. Am. J. Path 142:1952-1957).

The rapid and synchronous onset of apoptosis after CHB administration

overwhelms the capacity of duct cells, viable acinar cells and tissue macrophages to

rapidly remove apoptotic cells. As a consequence, most of the apoptotic cells remain

in situ undergoing progressive swelling, rupture of organelle and plasma membranes

5   and degradative change referred to as "secondary necrosis".

A feature of the CHB model of pancreatic involution is the limited

fragmentation of apoptotic acinar cells compared with that seen, for example, after

duct ligation (Walker 1987 supra). In the first hours after CHB administration and at

lower doses, apoptosis proceeds to cell fragmentation with intraepithelial

10   macrophages at 12 hours engorged with phagocytosed apoptotic bodies making it

unlikely that CHB prevents microfilament reorganisation.

Despite early cell death and edema, inflammatory cell infiltration is delayed,

reaching moderate density only at 48 hours, the number of neutrophils remaining

small throughout. In contrast, cerulein excites a vigorous inflammatory response

15   (Walker et al. 1993 supra; Fujimoto et al 1997 supra).

Acinar cell regeneration is limited to a few acini 10-18 days after CHB

administration. After cerulein and ethinione administration, it is rapid and complete

once the causative agent is removed (Fitzgerald 1960 Lb. Invest. 9:67-85; Isasser et

al. 1986 Pancreas 1:421-429).

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2.   Determination of Differences in Effect with Oral and Subcutaneous

Administration

2.1   Method..

Eight groups of eight male Wistar rats of approximately 200g were divided into 6 test

25   animals and 2 controls. Control animals were given either water orally by gavage or

saline subcutaneously as appropriate to their group. Test animals in each group were given CHB daily for 4 days, with doses for groups 50, 100, 150 and 200mg, orally by gavage in water or subcutaneously in saline. A further group was treated later with 130mg/kg subcutaneously in order to further define the dose-effect.

5   Animals were weighed each day and the day's dose of CHB calculated accordingly. Twenty-four hours after the fourth dose animals were euthanased using 60mg intra-peritoneal pentobarbitone and a full autopsy performed. Organs were removed and weighed before routine processing for histological examination. These were pancreas, brain, thymus, lungs, heart, salivary glands, lumbar vertebrum, paraspinal

10   skeletal muscle, pancreas, liver, intestine, kidney, spleen, seminal vesicle, testicle and prostate.

2.2   Results:

General

15   Three rats receiving 200mg/kg/day orally died after two doses, the remaining three test and two control animals were euthanased the same day. Three rats receiving 200mglkg/day subcutaneously were dead after one dose, necessitating the euthanasia of the rest of the group. One rat in the 100mg/kg oral test group died after one dose. Overall body weight gain was reduced for all test animals, those

20   animals receiving 130, 1 50 and 200mg/kg/day losing weight daily. Control animals gained 5 grams per day, rats given 50 and 100mg/kg/day gained 1-4 grams per day, rats given 130, 150 and 200 mg/kg/day lost weight. Pancreas and liver weights were not recorded. Spleen, thymus and lung weight was reduced in test animals, more so in high-dose and subcutaneous groups. Kidney weight was unchanged and testicle

25   weight as a proportion of body weight rose.

In the 200mg/kg s/c rats found dead there was haemorrhagic change in liver,

bowel, and under claws, pancreatic swelling and erythema, fat necrosis in the

abdomen and ascites. One rat in the 150mg/kg oral group had liver infarction and

bleeding in the thoracic and abdominal cavities.

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Pancreas

The effect of CHB on the pancreas was found to be dependent on both dose and

route. As with bodyweight, the subcutaneous route has a stronger effect. There was

considerable variability among the rats and within each pancreas.

10   Oral administration: Rats given 200mg/kg showed edema and moderate

apoptosis at 24 hours after their second dose. Other groups were examined 24

hours after the fourth daily dose. The 150mg/kg group showed less effect with mild

to moderate apoptosis, and occasional mitosis of acinar cells. With 100mg/kg, there

was mild apoptosis of acinar cells but conspicuously increased mitotic activity. The

15   same was true of the 50mg/kg group, with apoptosis less obvious.

Subcutaneous administration: Rats given CHB subcutaneously showed a

greater effect on the pancreas for the same dose. In the group given 200mg/kg, the

three rats which were dead at 24 hours all had marked Pancreatic edema and

moderate apoptosis. The pancreatic lobules were intact. In the three survivors which

20   were euthanased at 24 hours there was apoptosis of approximately 80% of acinar

cells with progression to secondary necrosis. In the 150mg/kg group, the effect 24

hours after 4 daily doses was almost complete loss of acinar cells, with lobules

composed of ductal complexes, mononuclear inflammatory cells and islets. Both

apoptosis and mitosis was present in ductal complexes and perilobular fibrosis was

25   present. In four of the six rats a layer of cells was present around the outer edge of

islets. At 130mg/kg, the same process of acinar cell loss occurred but was less

complete, about one-third of acinar cells surviving, and mitosis of acinar cells was

seen. Inflammation and mononuclear cell infiltrate were as marked as at the higher

doses. Peri-insular ductal cells were also present at this dose. At 100mg/kg, acinar

5   cell loss was about 20 – 80% with greater variability between rats and within

pancreases. 50mglkg caused only mild apoptosis but mitosis was increased.

Other Organs

Of other organs examined only the liver showed significant changes. The effect on

10   the liver was greater when CHB was given by the oral route.

Oral CHB: In the three rats that died after 200mg/kg all had widespread

necrosis of the liver. Nuclear changes were those of apoptosis with chromatin

condensation and fragmentation. Hepatocytes were necrotic and there were patches

of haemorrhage. In the three rats that survived until euthanasia, one rat had the

15   same degree of liver damage, another had focal inflammatory foci and loss of

hepatocytes, another had fine vacuolation of most hepatocytes with apoptotic bodies

in sinusoids.

150mg/kg orally induced liver changes in half the rats with focal loss of

hepatocytes, necrotic patches and clusters of sinusoidal cells in portal areas of the

20   lobule. Lower doses had no discernible effect.

Subcutaneous CHB: Four daily doses of 150mg/kg induced changes of liver

necrosis in subcapsular areas in contact with pancreas. The remainder of these livers

were normal.

25   2.3   Discussion

The effect of CHB on the pancreatic acinar cell is dose-related. The lesser

effect of the same dose by oral route is not surprising, attributable to less complete

absorption from the GIT and/or metabolism and neutralization in the liver as a first-

pass effect. When 200mg/kg is given orally in water it has a more toxic effect than

5   when in a corn-oil vehicle, causing fatal liver necrosis in half the animals and severe

hepatocyte damage in the survivors. Even at 150mg/kg orally, liver damage was

significant with only mild-moderate pancreatotoxicity. It is expected that absorption of

a water solution would be more complete and rapid than that of a suspension in oil.

Liver necrosis was evenly widespread. In the group given 150mg/kg subcutaneously,

10   liver necrosis was confined to subcapsular areas in contact with pancreas,

suggesting a local effect possibly due to enzyme action. The effect on the pancreas

of this hepatotoxic-dose is moderate. In a study of the pancreatotoxic effects of CHB

given in three vehicles (Wallig et al, 1989), the liver was not studied. If CHB is to be

used for its pancreatotoxic effects, then the oral route should be avoided.

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3.   *In Vivo* **effect of CHB on Pancreatic Carcinoma**

3.1   Method

Athymic rats 200 –250g were purchased from the Animal Resources Centre, Western

Australia. Four experiments were performed using slightly different doses with the

20   intention using a dose just sub-lethal in order to assess maximal effect.

Experiment 1: *Ductal* carcinoma cells (2×10^6 cells of Capan 2) were injected

into the left flank of 7 nude rats. Sixty days later, tumour nodules were 1 –2cm

diameter. By the time tumours were ready for testing, rats weighed approximately

300g. 135mg/kg CHB (absolute dose 38 - 40mg) was given into ventral abdominal

25   subcutaneous tissue in 0.5ml sterile saline with 2 controls getting saline only. At 18

hours, all test rats were dead. Autopsies were performed taking specimens of

carcinoma, pancreas and liver for processing.

Experiment 2: 6 rats were inoculated in the right flank with 5×10^6 cells of

5   Ar42J rat *acinar_cell* carcinoma. By 13 days all grew tumours 1 – 2cm diameter.

CHB at a dose of 125mg/kg mixed in 0.5ml saline (absolute dose 26 – 32.5mg) was

injected into ventral abdominal skin of all 6 rats. In this experiment there were no

controls. All rats survived until eighteen hours later when they were euthanased

using 60mg intraperitoneal pentabarbitone. Carcinoma, pancreas and liver were

10   dissected out and placed in formalin for processing.

Experiment 3: 14 rats were inoculated with 5×10^6 cells of Ar42J *acinar* cell

carcinoma. Seven rats were given CHB at 140mg/kg (approximately 30 – 32mg

absolute) and another 7 rats were given saline only. At 18 hours all rats had survived

15.   and were euthanased, pancreas, liver and carcinoma removed and taken for

processing.

3.2   Results

Experiment 1: *Ductal* carcinoma nodules were not effected by CHB.

20   Histological appearance was the same in both test and control groups.

Experiment 2: At lower doses of CHB, 1 of 6 test rats had evidence of a

cytotoxic effect with widespread apoptosis and secondary necrosis. Apoptosis was

as described previously, with crescentic clumping of chromatin, as well as wheel-rim

25   clumping around the nuclear membrane. Fragmentation of cells was not obvious as

described in other settings of massive synchronous apoptosis. In order to distinguish
the effect of CHB from patchy haemorrhagic necrosis, an effect was regarded as
present only if little viable tumour remained.

5   Experiment 3: Control tumour nodules were composed of sheets of uniform
large cells with dilated vascular channels and some areas of haemorrhage and
necrosis (Figure 6A and B, Figure 7A). Patches of apoptotic cells were present in
places, particularly near haemorrhages. Mitotic rate varied but in places was very
high. Three of 7 rats had a marked effect (Figure 6C and D, Figure 7B), a further 2
10   had about half surviving and the remaining 2 rats had no discernible effect.

Three test rats with acinar cell carcinoma that died of CHB toxicity in preliminary
dose-testing experiments had almost total cell death in the tumour nodules. The fact
that one rat had some patches of surviving tumour cells makes post-mortem change
unlikely to be responsible for the apoptotic appearance.

15   The effect on pancreas was different in athymic and Wistar rats (Figure 7C and
D). Athymic rats had a regional effect in the pancreas with areas of secondary
necrosis juxtaposed with well-preserved areas of acinar tissue, albeit with pyknotic
nuclei.

20   3.3   Discussion:

There is no discernible effect of CHB on ductal cells in the normal pancreas. It
is therefore not surprising that malignant ductal cells are not substantially affected by
CHB. Normal acinar cells, however, are sensitive and can be eliminated by a single
subcutaneous dose of 140mg/kg in the Wistar rat. Only very limited regeneration of
25   acinar cells was seen at 28 days, and this was in the peri-islet areas.

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The usual picture of apoptosis is that nuclear changes are followed rapidly by cytoplasmic condensation, blebbing and fragmentation. As described in other settings of massive synchronous apoptosis fragmentation was not obvious.

In the nude rat tumour nodules the effect of CHB appeared to be total regional

5   apoptosis or nothing. No part effect was seen. At the lower dose, 1 of 6 rats had an almost total cell kill, 5 had no discernible effect. At the higher dose, 3 of 7 had a marked effect with few areas of viable cells, and a further 2 of 7 had apoptotic change in about half the tumour section. In unaffected tumours, there was no increase in apoptosis and no decrease in mitosis. Areas of tumour were either totally

10   apoptotic or seemingly unaffected.

The effect on pancreas in the athymic rat is different from that in the Wistar rat. Despite marked edema as expected, the appearance of the pancreas is unusual in that nuclei in both necrotic and well-preserved areas are pyknotic. Patches of apoptotic cells are seen in the liver. It may be that a thymic humoral component is

15   involved in the widespread apoptotic process in Wistar pancreas, without which the lesion is different.

An advantage of the current invention is that the method of treatment can be used to kill acinar carcinoma cells in the pancreas and spread throughout the body. Acinar carcinoma cells are resistant to radiation treatment and chemotherapy. While

20   it may be possible to surgically remove the pancreas and hence the acinar carcinoma cells in the pancreas, detection of acinar cell carcinoma usually only occurs after the acinar carcinoma cells have spread from the pancreas. Furthermore the treatment only killed the acinar cells, and other cells in the pancreas appeared to be biologically functional after the treatment. Thus a patient would possibly avoid becoming diabetic

with functionally active islet cells in the pancreas. With surgical removal of the

pancreas a patient becomes diabetic and insulin must be administered.

It will of course be realised that while the foregoing has been given by way of

illustrative example of this invention all such and other modifications and variations

5   thereto as would be apparent to persons skilled in the art are deemed to fall within

the broad scope and ambit of this invention as defined in the claims appended

hereto.